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The *Escherichia coli* glycophage display system

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We describe a phage display technique that allows the production and selective enrichment of phages that display an *N*-glycoprotein (glycophages). We applied glycophage display to select functional glycosylation sequons from a pool of randomized acceptor sequences. Our system provides a genetic platform to study and engineer different steps in the pathway of bacterial *N*-linked protein glycosylation.

Keywords: *N*-glycosylation/oligosaccharyltransferase

Introduction

Asparagine-linked (*N*-linked) protein glycosylation, the most frequent posttranslational modification of secretory proteins in eukaryotic cells, was described to occur also in archaea (Yurist-Doutsch et al. 2008) and in the ε-proteobacterium *Campylobacter jejuni* (Szymanski et al. 1999). In the latter case, it was possible to transfer the glycosylation machinery that is encoded by a single locus, *pgl*, into *Escherichia coli*, resulting in glycosylation-competent *E. coli* cells (Wacker et al. 2002). This transfer into a genetically tractable system made it possible to study the basic mechanisms of bacterial *N*-linked protein glycosylation in a defined system (Feldman et al. 2005; Alaimo et al. 2006; Kowarik et al. 2006) and opens the possibility to apply advanced genetic tools, such as phage display, to study this pathway. The M13 phage display is a widely used in vitro selection technique that allows enrichment of proteins with desired binding specificity or catalytic activities from complex libraries (Smith and Petrenko 1997; Forrer et al. 1999; Tanaka et al. 2005). Phage display systems are based on genetic fusions of proteins or peptides to phage coat proteins. The fusion proteins are secreted to the periplasm (Steiner et al. 2006), where they stay membrane associated until they are incorporated into the progeny phage particle. Packaging of the fusion-encoding phagemid into the phage particles establishes a physical link between the phenotype of the displayed protein and the corresponding genotype. Recently, the scope of phage display was extended towards the display of posttranslational modifications, such as phosphorylation and phosphopantetheinylation (Yen and Yin 2007), and of proteins with reactive, unnatural amino acids that allow for subsequent site-specific conjugation with non-peptide ligands (Tian et al. 2004). We describe the combination of a phage display system with the process of *N*-linked protein glycosylation.

Results

To establish a novel genetic system of *N*-glycosylation in *E. coli*, we took advantage of the periplasmic co-localization of this protein modification and phage assembly to produce phage populations that expose an *N*-linked glycan on their surface (glycophages). The phenotypic display of *N*-linked glycans can be physically linked to any of the genes encoding components of the *N*-glycan biosynthetic pathway by locating the gene of interest on the phagemid (Figure 1).

In our initial setup, we constructed phagemid pHEN1(AcrA-P3) expressing the model glycoprotein AcrA of *C. jejuni* lacking the N-terminal lipid anchor sequence as a C-terminal fusion with the minor phage coat protein P3 (AcrA-P3). The pectate lyase B signal sequence (*pelB*) upstream of the *acrA* coding sequence ensured Sec-dependent translocation of the fusion protein. AcrA is glycosylated at asparagine residues N123 and N273 that are included within the bacterial consensus sequence D/E–X_1–N–X_2–S/T ($X_{1,2}\neq$P) (Kowarik et al. 2006). Non-glycosylatable AcrA(N123L, N273L)-P3 was expressed from phagemid pHEN1(AcrA*-P3) and served as a control. As expression host, we employed *E. coli* TG1 cells containing either plasmid pACYC^{Kan}(*pgl*) or pACYC^{Kan}(pgl_{mut}). Both constructs encode the complete operon for *N*-linked protein glycosylation from *C. jejuni*, the latter with a dysfunctional oligosaccharyltransferase PglB (Wacker et al. 2002). Superinfection of these cells with VCS M13 helper phage resulted in the packaging of the pHEN1 phagemids into progeny phage particles. Recombinant phage titers of ~10^{11} infective units per milliliter of culture supernatant were obtained. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation of cell-free total phage protein followed by immunoblot analysis identified a protein that was cross-reactive against both P3 antibody and anti-AcrA antiserum and migrated as a protein of ~120 kDa molecular mass, corresponding to the expected mobility of AcrA-P3 (Figure 2). The lack of this protein in the culture supernatant from non-infected cells demonstrated

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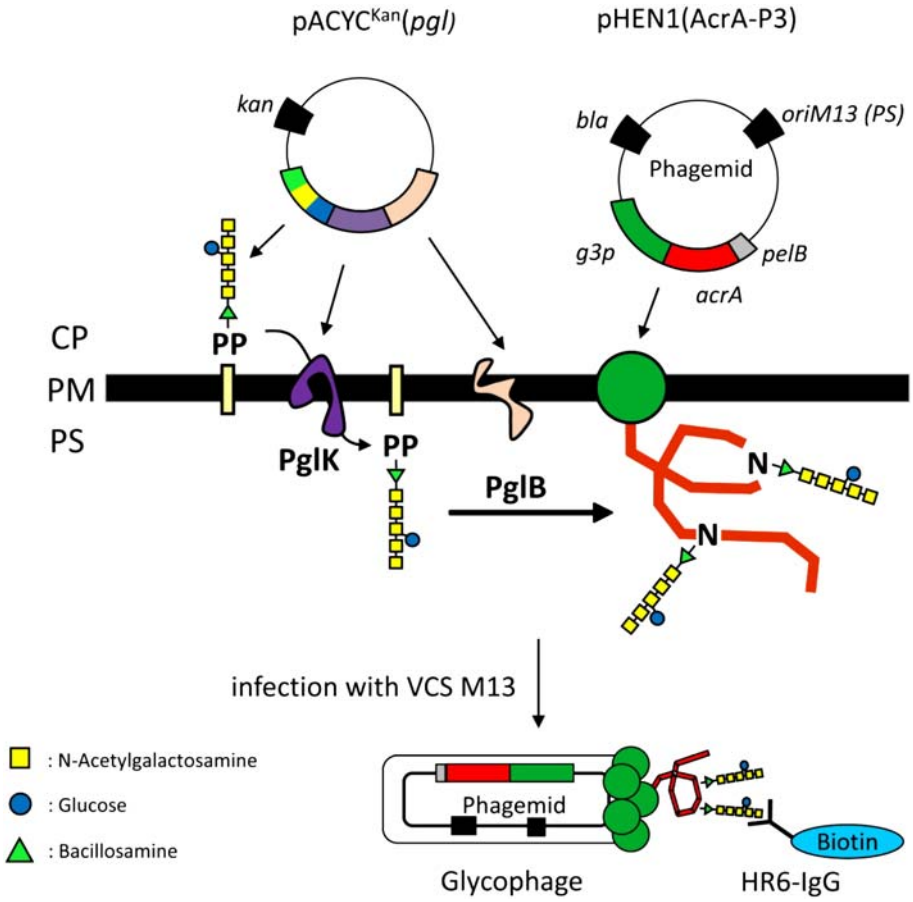

Fig. 1. The glycophage display system. Plasmids/phagemids encoding the enzymes for *N*-linked protein glycosylation (pACYC^{Kan}(*pgl*)) and for the acceptor AcrA-P3 fusion protein (pHEN1(AcrA-P3)) are shown. Catalyzed by individual glycosyltransferases, the oligosaccharide is assembled on a lipid carrier, bactoprenylpyrophosphate, at the cytoplasmic side (CP) of the inner membrane. The lipid-linked glycan is translocated by the flippase PglK across the inner membrane (IM) to the periplasmic space (PS) where the glycan is transferred to specific asparagine residues (N) of the acceptor protein AcrA-P3 by the oligosaccharyltransferase PglB. After infection with helper phage VCS M13, secreted phage particles that display the glycosylated acceptor protein (glycophages) are bound to glycan-reactive, biotinylated IgG, and the resulting immunocomplex is captured with streptavidin-coated beads. Glycophages are finally eluted and used to re-infect *E. coli* (F+) cells. In general, the glyco-phenotype of the phage can be connected to the genotype for any enzyme required for bacterial, *N*-linked protein glycosylation (*kan*, kanamycin resistance gene; *bla*, gene encoding beta-lactamase).

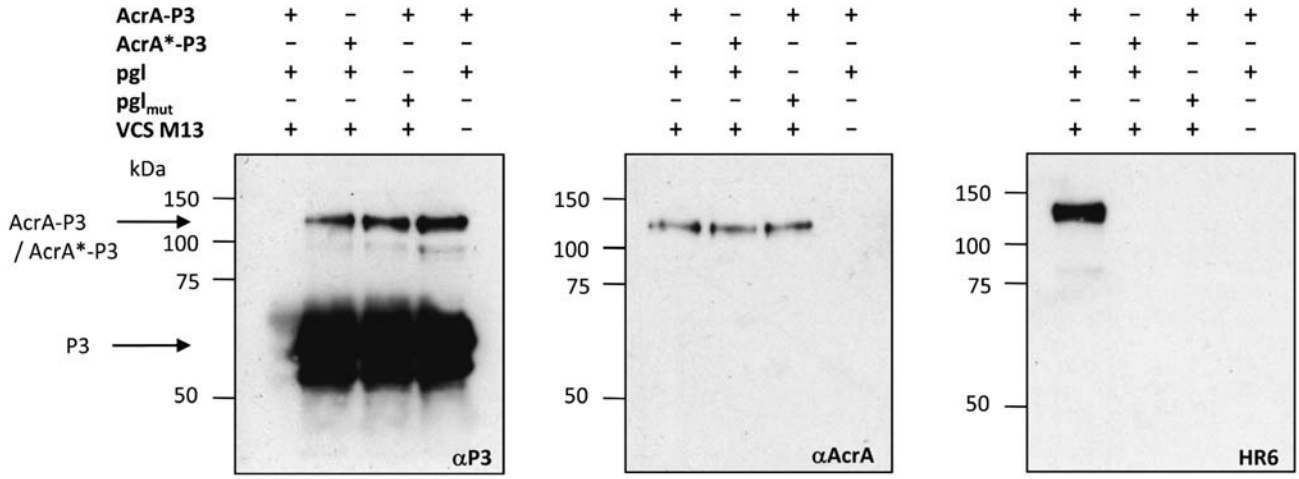

Fig. 2. Immunoblot analyses of phage samples. Phages produced from glycosylation-competent *E. coli* TG1 (*pgl*) cells, harboring either phagemid pHEN1 (AcrA-P3) or pHEN1(AcrA*-P3), and from non-glycosylation-competent *E. coli* TG1 (*pgl*$_{mut}$) cells, harboring phagemid pHEN1(AcrA-P3), were separated by SDS-PAGE. Phages were collected by PEG precipitation of cell-free culture supernatants, and sample amounts were normalized to OD_{268}. The signal intensity of the P3 wild-type protein at ~60 kDa reflects equivalent amounts of phage protein per sample. P3, AcrA-P3, AcrA*-P3, and glycosylated AcrA-P3 were visualized with monoclonal anti-P3 antibody, polyclonal AcrA-, and glycan-specific antiserum HR6.

phage-dependent secretion to medium. To detect glycosylated AcrA-P3, the same phage samples were probed with glycosylation-specific HR6 antiserum that had been raised against *E. coli* membranes displaying the *C. jejuni* glycan (S. Amber and M. Aebi, manuscript in preparation). Here, an immunoreactive protein with a mass of ~120 kDa was detected in phage samples that were derived from TG1(*pgl*, AcrA-P3) cells. In contrast, this signal was absent when either TG1(*pgl*, AcrA*-P3) or TG1(pgl_{mut}, AcrA-P3) cells were used for phage preparation. These results demonstrated that fusion proteins AcrA-P3 and the AcrA*-P3 were both translocated to the periplasm of *E. coli* TG1 and that only the former was glycosylated by the enzymatic machinery encoded by the *C. jejuni pgl* operon.

To make use of the display of glycosylated AcrA-P3, we developed a panning strategy that allowed selective enrichment of glycophages. We isolated and biotinylated the IgG fraction from the anti-glycan reactive HR6 antiserum and mixed it with glycophage containing phage preparations. The resulting IgG/glycophage immunocomplex was captured with streptavidin-coated beads. Bound phages were eluted by a shift to basic conditions (pH 12). To determine the efficiency of this panning method, we aimed to enrich glycophages from excess background of phages displaying non-glycosylated AcrA*-P3. Glycosylation-competent *E. coli* TG1 (*pgl*) cells expressing either glycosylatable AcrA-P3 or non-glycosylatable AcrA*-P3 were mixed in ratios from 1:10^3 to 1:10^6. After superinfection of the mixed cultures with helper phage progeny, phage samples were prepared and subjected to the HR6-IgG panning procedure. Eluates containing enriched phage populations were used to re-infect glycosylation-competent *E. coli* TG1 (*pgl*) cells. Pooled re-infectants were used for additional HR6-IgG panning cycles. In addition, these phage preparations were examined by immunoblot analysis to identify glycosylated AcrA-P3. Phage samples that were produced from cultures with a 10^3-, 10^5-, and 10^6-fold excess of cells expressing non-glycosylatable AcrA*-P3 showed a HR6 reactive signal for glycosylated AcrA-P3 after one, two and three rounds of enrichment, respectively (Supplementary Figure 1), confirming selective enrichment of glycosylatable phagemid. To measure the enrichment of the glycophage-associated genotype, the relative increase of re-infectants containing phagemid pHEN1(AcrA-P3) was monitored after each panning cycle by polymerase chain reaction (PCR) (Table 1 and Materials and methods section). Phagemid pHEN1(AcrA-P3) was enriched up to 100-fold per round of panning. Consequently, three and four cycles of our panning method were sufficient to convert mixed populations with a 10^5- and 10^6-fold excess of non-glycophage-producing cells into almost homogenous populations of glycophage-producing cells, respectively (Table 1 and Supplementary Figure 1).

To evaluate the potential of the glycophage system for the selection of glycosylation-specific genetic traits, we investigated the sequence requirements of a bacterial *N*-glycosylation site as a proof of principle. The bacterial *N*-glycosylation requires a glutamate or aspartate residue at the −2 position relative to the asparagine in the consensus sequence N–X–S/T(Kowarik et al. 2006). We created a library of AcrA(N123, N273L)-P3 in which the residues at positions −2, −1, and +1 relative to the single glycosylation site N123 of this AcrA variant were randomized, N123 was kept constant, and only serine or threonine residues were allowed at position +2. The corresponding mutations were introduced into pHEN1(AcrA*-P3) by PCR using degenerated oligonucleotides. The library with a theoretical complexity of 1.6 × 10^4 AcrA variants consisted of about 1.5 × 10^5 independent clones and thus essentially covered the theoretical sequence space. The complexity of the library was verified by DNA sequence analysis to ensure the random distribution of all four nucleotides for the codon triplet at positions −2, −1, and +1 of the glycosylation site, while A or T varied at the first position of the triplet codon for the +2 position (corresponding to S or T). Three panning rounds were carried out with phages displaying the degenerate AcrA-P3 library. Phagemids from single colonies obtained after infection of *E. coli* TG1(pACYC(*pgl*)) cells with the eluates after each panning round were assessed by DNA sequencing. The deduced amino acid sequences of the randomized AcrA-P3 segments from clones encoding full-length AcrA-P3 variants are listed in Table 2. Within this phagemid population, the proportion of variants encoding an aspartate (D) or a glutamate (E) at position −2 relative to N123 was continuously increased during the course of the panning procedure, in agreement with the previously described requirement of the D/E–X_1–N–X_2–S/T sequence for *N*-glycosylation in this bacterial system(Kowarik et al. 2006). Further analyses of the amino acid distribution at the −2 and +2 positions showed that aspartate was favored over glutamate and serine favored over threonine, respectively. A slight preference for hydrophobic residues could be observed at positions +1 or −1 but without enrichment of specific hydrophobic amino acids.

Table 1. Glycophage enrichment

E. coli TG1 (*pgl*) cells (OD_{600} equivalents)		Enrichment of the glyco-genotype (pHEN1(AcrA-P3)) after the indicated round of HR6-IgG panning (clones with glycosylation-competent genotype/number of analyzed clones)			
AcrA-P3	AcrA*-P3 (N123L, N273L)	1st	2nd	3rd	4th
10^{-3}	1	6/16	15/16	–	–
10^{-5}	1	0/14	11/32	16/16	–
10^{-6}	1	–	0/16	2/23	22/23

Glycosylation-competent *E. coli* TG1 (*pgl*) cells expressing either glycosylatable AcrA-P3 or non-glycosylatable AcrA*-P3 were mixed at the indicated ratios. After infecting the mixed cell cultures with VCS M13, progeny phages were prepared and applied to multiple rounds of the HR6-IgG-biotin panning. Phages containing eluates obtained after each panning cycle were used to re-infect *E. coli* TG1 (*pgl*) cells. Ampicillin-resistant re-infectants were then screened (see Materials and methods section) to determine the proportion of clones containing the glycosylation-competent pHEN1(AcrA-P3) phagemid.

Discussion

We extended the analysis of the established phage display technology towards the posttranslational process of *N*-linked protein glycosylation. The glycophages displayed the heptaoligosaccharide of *C. jejuni*, which is also produced in *E. coli*. Using glycan-specific HR6 antibodies, we developed a high-affinity selection procedure and showed that glycophages were still infective towards the *E. coli* F^+ strain TG1 after elution. The enrichment of glycophages was possible from mixed cell cultures where 10^3 glycophage-producing cells were mixed with

Table 2. Enrichment of functional glycosylation sites

Randomly selected clones from initial library pool AcrA(X-X-N_{123}-X-S/T)-P3	Randomly selected clones from enriched pools after the indicated round of HR6-IgG panning		
	1st	2nd	3rd
VLNCS	TSNSS	ELNLS	DNNTS
RWNWT	TANNT	NPNFT	DLNMS
MLNTS	GFNGT	IVNIT	DCNKS
LYNGS	RYNFS	DDNVS	DNNRS
AINLS	SSNQS	EVNDS	DHNMS
NANTS	MGNRT	ENNAS	DINSS
LNNTS	QLNLT	EGNRS	DYNVS
SHNIT	AGNRS		DINSS
ANNQT	IHNDS		DFNLT
YSNRT	EVNVT		DDNVS
GGNFS	GGNMS		DFNLT
	GLNCS		IKNKS
			DLNVT
			ENNAS
			EVNSS
			DANVS
			DLNVT
			DGNPS
			TDNIT
			ARNVS
			ETNAT
			DTNTS
			ECNLS
N_{total}=14	**N_{total}=17**	**N_{total}=27**	**N_{total}=59**
N_{Δ}= 3	**N_{Δ}= 5**	**N_{Δ}= 20**	**N_{Δ}=36**
N_{Δ}/N_{total}=0.21	**N_{Δ}/N_{total}=0.29**	**N_{Δ}/N_{total}=0.74**	**N_{Δ}/N_{total}=0.61**

Pentapeptide sequences surrounding the randomized AcrA glycosylation site N123 were obtained before and after several rounds of enrichment. The sequences of library variants encoding a full-length fusion protein are shown. Sequences representing the canonical glycosylation site motif for bacterial *N*-linked glycosylation are highlighted in gray. N_{total}, number of analyzed clones, N_{Δ}, number of clones containing a stop codon, a frame shift, or a deletion.

10^6 non-glycophage-producing cells prior to infection with helper phage. In contrast to classical phagemid-based display systems where the number of recoverable phages depends mainly on the level of protein display, with typically 1% to 10% of recombinant phages displaying the phagemid encoded protein (Clackson and Lowman 2004), the formation of glycophages additionally requires enzymatic modification of the fusion protein prior to its incorporation into phage particles. Both effects, incomplete glycosylation and disfavored incorporation of the fusion protein, considerably reduced the fraction of recoverable phages. As less than 0.1% of the phages produced from infected TG1(*pgl*, AcrA-P3) cells were glycophages, our data reveal glycophage enrichment from an at least 10^9-fold excess of non-glycophages produced from a mixed cell culture.

The identification of the specific requirements for bacterial *N*-glycosylation using the glycophage display system in an acceptor site screen presents a proof of concept. However, it is important to note that the enrichment of clones with functional glycosylation sites was partly masked by the accumulation of clones containing a stop codon, a frame shift, or a deletion upstream of the g3p gene which consequently abolished translation and display of the fusion protein. The enrichment of such clones was most likely favored at two levels during the selection pro-

Table 3. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pHEN1	Phagemid with the M13 origin of replication, *Nco*I/*Not*I cloned inserts are expressed as N-terminal fusions with the P3 phage coat protein with a *pelB* leader sequence for translocation. Expression is controlled by the *lacZ* promoter, Amp^R	(Hoogenboom et al. 1991)
$pACYC^{Kan}$(*pgl*)	Encodes the *C. jejuni pgl* cluster, Kan^R	This work
$pACYC^{Kan}$(pgl_{mut})	Encodes the *C. jejuni pgl* cluster containing mutations W458A and D459A in PglB, Kan^R	This work
pHEN1(AcrA-P3)	Phagemid expressing glycosylatable AcrA-P3 fusion protein under the control of the *lac* promoter with *pelB* leader sequence, Amp^R	This work
pHEN1(AcrA*-P3)	Phagemid expressing non-glycosylatable AcrA(N123L, N273L)-P3 fusion protein under the control of the *lac* promoter with *pelB* leader sequence, Amp^R	This work
para(AcrA-P3)	Phagemid expressing AcrA-P3 fusion protein under the control of p_{BAD} promoter with *pelB* leader sequence, Amp^R	This work (Supplementary information)
para(AcrA(N123L,N273L)-P3)	Phagemid expressing AcrA (N123L, N273L)-P3 fusion protein under the control of p_{BAD} promoter with *pelB* leader sequence, Amp^R	This work (Supplementary information)

cess. Firstly, the corresponding cells are not impaired by the expression of the potentially inhibitory heterologous fusion protein, and secondly, the phages produced from such clones have a wild-type phenotype and thus confer their genetic information much more efficiently than those displaying a bulky fusion protein during re-infection. The enrichment of phagemids that did not express the fusion protein occurred only when we used a randomized phage library; such phagemids were not observed in our reconstitution experiments using phages that express either glycosylatable or non-glycosylatable AcrA-P3 fusion protein. We postulate that such phages were generated in the course of the library preparation.

The glyco-epitope used in our experimental setup is the product of a biosynthetic pathway that requires multiple components: cytoplasmic glycosyltransferases of the oligosaccharide biosynthetic pathway, the lipid-linked oligosaccharide flippase, the oligosaccharyltransferase, and the acceptor protein. The glyco-phenotype of the phage can therefore be directly linked to the genotype of any of the steps required for the synthesis of the glyco-epitope displayed on the surface of the filamentous phage M13 (Figure 1), and it can be used for the functional screening of variations in each of these steps. The glycophage screening system might therefore be exploited for the identification of novel oligosaccharyltransferases and flippases, the identification of new acceptor peptides, or the identification of novel glycosyltransferases involved in the assembly of lipid-linked oligosaccharides. In the latter case, the enrichment strategy needs to be modified to allow for the selection of the target glycan structure, e.g., by using specific lectins or antibodies. These screening procedures are possible due to the relaxed lipid-linked oligosaccharide specificity of the key elements in the *N*-glycosylation pathway, the lipid-linked oligosaccharide flippase PglK(Alaimo et al. 2006), and the oligosaccharyltransferase PglB(Feldman et al. 2005). In addition, in vitro evolution strategies to find pathway components with altered properties will now be possible.

Materials and methods

Strains and material

E. coli XL1-Blue (Stratagene) was used for cloning and *E. coli* TG1 (Stratagene) for production and titer determination of M13 phages. VCS M13 (Stratagene) was used as helper phage and propagated as described (Clackson and Lowman 2004).

Phagemid constructions

Molecular genetics methods were performed according to standard protocols (Sambrook and Russell 2001). To express wild-type AcrA fused to the minor phage coat protein P3, an *acrA*-containing PCR fragment was amplified from plasmid para(AcrA-P3) (Table 3; Supplementary information) and inserted into *Nco*I–*Not*I cut phagemid pHEN1 (Hoogenboom et al. 1991). The phagemid backbone pHEN1 has the M13 *ori* to allow the phagemid to be packaged into the phage particles and encodes ampicillin resistance for selection. Primers NcoI_fw (5′-AGCCGGCGATGGCCATGGATATTGGAATTAATTCG-3′) and NotI_rv (5′-TGTCGTCGTTGGCGGCCGCTTGTGC-TCCAATTTC-3′, the *Nco*I and the *Not*I restriction sites are underlined, respectively) were used for amplification from template vector para(AcrA-P3) (Supplementary information). On the resulting phagemid pHEN1(AcrA-P3), the gene for the fusion protein is transcribed under control of the *lacZ* promoter/operator and expressed with the N-terminal peptide leader sequence *pelB* to direct Sec-dependent translocation to the periplasm. A phagemid encoding non-glycosylatable AcrA (N123L, N273L)-P3 was constructed by replacing the *Nco*I-*Eag*I fragment of phagemid pHEN1(AcrA-P3) with the *Nco*I-*Eag*I fragment of phagemid para(AcrA(N123L, N273L)-P3) (Supplementary information) resulting in pHEN1(AcrA*-P3).

To randomize the positions −2, −1, and +1 and to allow a serine or threonine insertion at position +2 of the AcrA wild-type glycosylation sequon N123, a 783-bp PCR fragment was created with primers NF_1 (5′-TAACTTTCGAAAATGCAAG-CAAGNNNNNNAAYNNNWCTAAAGCTCTTTTTAGC-3′) and AcrA_pHEN_2 (5′-AATCGTCATCTGCGGCCGCTT-GTGCTCCAATTTCTTTAACTTCG-3′, the *Bsp*119I and the *Eco*52I restriction sites are underlined, respectively) using phagemid pHEN1(AcrA(N123L, N273L)-P3) as template. The PCR product was used to replace the *Bsp*119I-*Eco*52I fragment from phagemid pHEN1(AcrA(N123L, N273L)-P3), resulting in the phagemid library pHEN1(AcrA(X-X-N_{123}X-S/T)-P3).

Production of phages displaying glycosylated AcrA (glycophages)

2xTY medium containing 1% glucose, 100 μg/mL ampicillin, and 35 μg/mL kanamycin were inoculated with a single colony of *E. coli* TG1 harboring the phagemid pHEN1(AcrA-P3) and the *pgl* operon encoded by plasmid pACYC(*pgl*) and grown

overnight at 37°C with shaking at 190 rpm. Fresh 2xTY medium (25 mL) containing 1% glucose, 100 μg/mL ampicillin, and 35 μg/mL kanamycin was inoculated with the overnight culture to OD_{600} of 0.1 and grown at 37°C with shaking at 190 rpm. Cells were superinfected with a helper phage preparation at OD_{600} of 0.5 at a multiplicity of infection of ~20, and the cultures were incubated for 40 min at 37°C without shaking. The medium was changed by harvesting the cells by centrifugation (3000 × *g*, 4°C, 10 min), and the cell pellet was resuspended in 50 mL 2xTY medium containing 100 μg/mL ampicillin and 35 μg/mL kanamycin. The cultures were grown overnight at 30°C with shaking at 190 rpm. After growth for 16 to 20 h, the culture supernatant was separated from the cells by centrifugation (3000 × *g*, 4°C, 30 min) and incubated on ice for 1 h after addition of a solution of ice-cold 20% polyethyleneglycol (PEG, Fluka)—2.5 M NaCl to one-fifth of the final volume. The precipitated phage particles were collected by centrifugation (3000 × *g*, 4°C, 30 min) and dissolved in 1 mL of water. To remove any HR6-reactive remnants from the phage surface, the phage particles were further purified by treatment with 1% Triton X-100 at room temperature (RT) for 30 min on a turning wheel (60 U/min). Phages were again PEG precipitated, redissolved in 1 mL phosphate-buffered saline (PBS), and further incubated with 2% sarkosyl at RT for 30 min on a turning wheel. After a final PEG-precipitation, phages were dissolved in 1 mL PBS containing 10% glycerol and a protease inhibitor cocktail (Complete, EDTA-free, Roche Diagnostics). The total concentration of phage particles was estimated spectrophotometrically at OD_{268} (with OD_{268} = 1.0 corresponding to $5*10^{12}$ colony forming units per milliliter (Clackson and Lowman 2004)). Phage titers were determined by infecting 0.9 mL of exponential phase *E. coli* TG1 cells with 100 μL of a 10^4-fold dilution of the phage preparation followed by incubation for 40 min at 37°C without shaking. One hundred microliters of a 10^2- and a 10^4-fold dilution of the infection sample were spread on 2xTY plates containing 1% glucose and 100 μg/mL ampicillin. The plates were incubated for 16 h at 37°C, and the plaques were counted.

Cell mixing experiment

To determine the minimal fraction of glycophage-producing cells that still allowed the trapping of glycophages by the HR6-IgG panning, glycophage-producing *E. coli* TG1 (pHEN1(AcrA-P3), pACYC(*pgl*)) cells were mixed with 10^3-, 10^5-, and 10^6-fold excesses of non-glycophage-producing *E. coli* TG1 (pHEN1(AcrA*-P3), pACYC(*pgl*)) cells (based on OD_{600} equivalents). Fresh 2xTY medium (25 mL) containing 1% glucose, 100 μg/mL ampicillin, and 35 μg/mL kanamycin was inoculated with each of the cell mixtures to OD_{600} of 0.1 and grown at 37°C with shaking at 160 rpm. Progeny phages were produced by superinfecting mixed cell populations with helper phage (as described above) and glycophages enriched by the HR6-IgG panning (see below).

Enrichment of glycophages

Approximately 10^{10} cfu of glycophage-containing phage preparations were mixed with 5 mL PBS containing 4% milk, 0.1% Tween-20, and 1.5 μg/mL biotinylated HR6-IgG (~6 mol biotin per mol IgG) and incubated for 1 h at RT on a turning wheel (30 U/min). Streptavidin-coated beads (50 μL) (binding capacity 2 mg/mL biotinylated BSA/mL; PIERCE) were washed two times with 10 mL PBS and blocked with 10 mL PBS containing 4% milk and 0.1% Tween-20 for 30 min on a turning wheel at RT. The blocked beads were sedimented (3000 g, 2 min) and added to the phage/HR6-IgG sample to capture the immunocomplex. After incubating the mixture for 1 h at RT, the beads were washed five times with 10 mL PBS containing 0.1% Tween-20 and five times with 10 mL PBS (for each washing step, beads were incubated 5 min at RT on turning wheel and then sedimented). To elute adherent glycophages, the beads were incubated with 1 mL of 100 mM triethylamine (pH 12.0) and sedimented, and the supernatant was immediately neutralized with 500 μL 1 M Tris-HCl (pH 7.2). To recover the phagemids from eluted phages for genotype determination, 0.1 mL of the neutralized eluate was used to infect 0.9 mL exponential-phase *E. coli* TG1 cells (as described above). To repropagate the enriched phage population, 1 mL of the neutralized eluate was used to infect 9 mL exponential-phase *E. coli* TG1 cells harboring the plasmid $pACYC^{Kan}$(*pgl*). The infected cells were harvested by centrifugation (2500 × *g*, 5 min), resuspended in 1 mL 2xTY medium, and spread on five 2xTY agar plates containing 1% glucose, 100 μg/mL ampicillin, and 35 μg/mL kanamycin. After incubating the plates for 16 h at 37°C, all colonies were pooled and used to inoculate 25 mL 2xTY medium containing 1% glucose, 100 μg/mL ampicillin, and 35 μg/mL kanamycin to OD_{600} of 0.1. Glycophages were produced from this culture as described above und subjected to further rounds of enrichment.

Genotype discrimination between phagemids pHEN1(AcrA-P3) and pHEN1(AcrA-P3)*

Phagemid DNA of pHEN1(AcrA-P3) and pHEN1(AcrA*-P3) can be discriminated by *Eco*RI digestion since the latter phagemid contains an additional *Eco*RI site due to the N273L mutation. To determine which phagemids were present in an *E. coli* TG1 reinfectant, a 1443-bp fragment was amplified using primer pair screen_fw (5′-AAGCGGAAGAGCGCCCAATACG-3′)/NotI_rv (sequence, see above) by colony PCR, and the DNA was analyzed by gel electrophoresis after *Eco*RI digestion. The *Eco*RI digested fragment amplified from phagemids pHEN1(AcrA-P3) resolved into diagnostic fragments of 368 and 1075 bp. In contrast, the fragments amplified from phagemid pHEN1(AcrA*-P3) resolved into fragments of 774, 386, and 301 bp after *Eco*RI digest.

Preparation of biotinylated HR6-IgG

Raw serum (rabbit) (500 μL) was diluted with 10 mL binding buffer (20 mM sodium phosphate, pH 7) and filtrated through a membrane with 0.22 μm pore size. To isolate the IgG fraction, the serum sample was subjected to protein G chromatography (HiTrap, GE Helthcare) according to the manufacturer's protocol. IgG containing eluates were analyzed by SDS-PAGE, and fractions containing pure IgG were combined. The pooled fractions were buffer exchanged to PBS using a PD-10 desalting column and used for in vitro biotinylation with Sulfo-NHS-LC-Biotin reagent (PIERCE) according to the manufacturer's protocol. The biotinylated HR6-IgG sample was again desalted using a PD-10 desalting column, and the final IgG concentration was adjusted to 0.5 mg/mL.

Phage immunoblots

Approximately 4×10^{10} purified phage particles per lane were applied to a 10% SDS-PAGE under reducing conditions, and the protein bands were subsequently electroblotted on a nitrocellulose membrane (Protran membrane, Whatman). The membranes were blocked for 1 h at RT in PBS containing 10% milk and 0.1% Tween-20. As primary antibody, either mouse anti-P3 (New England Biolab), rabbit anti-AcrA[1], or rabbit HR6 antiserum (S. Amber and M. Aebi, manuscript in preparation) was used in PBS containing 5% milk and 0.1% Tween-20 (1 h at RT). As secondary antibody, either a goat anti-mouse or goat anti-rabbit IgG horseradish peroxidase conjugate (Santa Cruz) was used in PBS containing 5% milk and 0.1% Tween-20 (1 h at RT). The horseradish peroxidase reactivity was detected with ECL reagent (GE Healthcare).

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Supplementary Data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

Abbreviations

PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PEG, polyethyleneglycol; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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